PB83-223693

Sensitive Assay, Based on Hydroxy Fatty Acids from Lipopolysaccharide Lipid A, for Gram-Negative Bacteria in Sediments

Florida State Univ., Tallahassee

Prepared for

Environmental Research Lab., Gulf Breeze, FL

16 Jul 82

U.S. Department of Commerce
National Technical Information Service
NTIS

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO. EPA-600/J-82-326	2.	3. PB83-223693
4. TITLE AND SUBTITLE SENSITIVE ASSAY, BASED ON HYDROXY FATTY ACIDS FROM LIPOPOLYSACCHARIDE LIPID A, FOR GRAM-NEGATIVE BACTERIA IN SEDIMENTS (Journal Version)		5. REPORT DATE 1982 6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) J.H. Parker, G.A. Smith, H.L. Fredrickson, J.R. Vestal, and D.C. White		8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS Department of Biological Science Florida State University Tallahassee, FL 32306		10. PROGRAM ELEMENT NO. 11. CONTRACT/GRANT NO. CR 807321
12. SPONSORING AGENCY NAME AND ADDRESS U.S. Environmental Protection Agency Environmental Research Laboratory Office of Research and Development Gulf Breeze, FL 32561		13. TYPE OF REPORT AND PERIOD COVERED 14. SPONSORING AGENCY CODE EPA/600/04

15. SUPPLEMENTARY NOTES
Appl. Environ. Microbiol. 1982. 44(5)1170-1177

16. ABSTRACT

Biochemical measures have provided insight into the biomass and community structure of sedimentary microbiota without the requirement of selection by growth or quantitative removal from the sediment grains. This study used the assay of the hydroxy fatty acids released from the lipid A of the lipopolysaccharide in sediments to provide as estimate of the gram-negative bacteria. The method was sensitive to picomolar amounts of hydroxy fatty acids. The recovery of lipopolysaccharide hydroxy fatty acids from organisms added to sediments was quantitative. The lipids were extracted from the sediments with a single-phase chloroform-methanol extraction. The lipid-extracted residue was hydrolyzed in 1 N HCL, and hydroxy fatty acids of the lipopolysaccharide were recovered in chloroform for analysis by gas-liquid chromatography. This method proved to be about fivefold more sensitive than the classicla phenol-water or trichloroacetic acid methods when applied to marine sediments. By examination of the patterns of hydroxy fatty acids, it was also possible to help define the community structure of the sedimentary gram-negative bacteria.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

18. DISTRIBUTION STATEMENT Release to public	19. SECURITY CLASS (This Report) unclassified	21. NO. OF PAGES 9
	20. SECURITY CLASS (This page) unclassified	22. PRICE

EPA-600/J-82-326
JOURNAL ARTICLE

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1982, p. 1170–1177 Vol. 44, No. 5
0099-2240/82/111170-08$02.00/0
American Society for Microbiology

Sensitive Assay, Based on Hydroxy Fatty Acids from Lipopolysaccharide Lipid A, for Gram-Negative Bacteria in Sediments

JEFFREY H. PARKER,[1] GLEN A. SMITH,[1] HERBERT L. FREDRICKSON,[1] J. ROBIE VESTAL,[2] AND DAVID C. WHITE[1]*

Department of Biological Science, Florida State University, Tallahassee, Florida 32306,[1] and Department of Biological Science, University of Cincinnati, Cincinnati, Ohio 45221[2]

Received 6 April 1982/Accepted 16 July 1982

Biochemical measures have provided insight into the biomass and community structure of sedimentary microbiota without the requirement of selection by growth or quantitative removal from the sediment grains. This study used the assay of the hydroxy fatty acids released from the lipid A of the lipopolysaccharide in sediments to provide an estimate of the gram-negative bacteria. The method was sensitive to picomolar amounts of hydroxy fatty acids. The recovery of lipopolysaccharide hydroxy fatty acids from organisms added to sediments was quantitative. The lipids were extracted from the sediments with a single-phase chloroform-methanol extraction. The lipid-extracted residue was hydrolyzed in 1 N HCl, and the hydroxy fatty acids of the lipopolysaccharide were recovered in chloroform for analysis by gas-liquid chromatography. This method proved to be about fivefold more sensitive than the classical phenol-water or trichloroacetic acid methods when applied to marine sediments. By examination of the patterns of hydroxy fatty acids, it was also possible to help define the community structure of the sedimentary gram-negative bacteria.

Biochemical methods have proved to be very useful in assessing the microbial biomass of sediments (10, 18, 29, 32). These methods are not dependent upon growth of the organisms for assay or on the quantitative removal of organisms from the sediments (10, 19). Estimations of the total cellular biomass are readily made from the extractable phospholipid (32). The procaryotic biomass can be unequivocally estimated from the muramic acid (10; R. H. Findlay, D. J. W. Moriarty, and D. C. White, Geomicrobiology J., in press). To translate the muramic acid content into cellular carbon, the proportion of cyanophytes, gram-negative, and gram-positive bacteria must be known (10, 18). The proportions of gram-negative and gram-positive bacteria in sediments have been determined by examining the cell wall structure with the electron microscope (20). An estimate of the gram-negative bacteria in sediments from an analysis of the lipopolysaccharide (LPS) has been provided by Saddler and Wardlaw (23). They extracted sediments with phenol-water or trichloroacetic acid and assayed the LPS by its content of ketodeoxyoctonate and β-hydroxymyristic acid, as well as its anticomplementary activity when tested with human serum. The present paper describes a method for the optimization of the assay of LPS from sediments, using a simpler extraction that is more sensitive and at the same time yields specific information on the gram-negative bacterial community structure.

The method in the present study utilizes an initial lipid extraction. Previous work has shown that analysis of the lipids can be used in studies of the community structure (2, 30). In this study, the lipid-extracted residue was subjected to mild acid hydrolysis, and the lipid A fatty acids were recovered. Purification and derivatization of the hydroxy fatty acids followed by analysis by gas-liquid chromatography (GLC) yields a profile which can be compared to the catalogues of components from bacterial monocultures (21, 35) and can perhaps be utilized to gain indications of the community structure as described by Mayberry et al. (17) for clinical specimens.

MATERIALS AND METHODS

Materials. Glass-distilled solvents (Burdick and Jackson, Muskegon, Mich.), freshly distilled chloroform, and derivatizing agents from Pierce Chemical Co., Inc., Rockford, Ill., Aldrich Chemical Co., Inc., Milwaukee, Wis., and PCR Research Chemicals, Inc., Gainesville, Fla., were utilized. Standard fatty acids, lyophilized cultures of *Escherichia coli* strain b, *Pseudomonas fluorescens*, *Bacillus subtilis* ATCC 6633, and *Clostridium welchii* ATCC 13124 were obtained from Sigma Chemical Co., Inc., St. Louis, Mo. *P. atlantica* was the gift of W. A. Corpe, Columbia

University, New York, N.Y., and was grown as described (11). *P. maltophilia* was the gift of T. G. Tornabene, Georgia Institute of Technology, Atlanta, Ga., and was cultured as described (25).

The groundwater sediment samples were recovered from the 12 to 13 ft (ca. 3.66 to 3.96 m) horizons below the surface at Ft. Polk, La., with special apparatus and precautions to prevent surface contamination designed by J. McNabb and M. R. Scalf of the R. S. Kerr Environmental Research Laboratory at Ada, Okla., and S. Hutchings of Rice University, Houston, Tex. Samples were frozen at −90°C at the well site and maintained frozen until analysis. Samples of estuarine sediments were recovered at the Florida State University Marine Laboratory (29°C 54′ N, 84° 37.8′ W), extracted in the field, and returned to the laboratory for analysis.

Authentic hydroxy fatty acids were the gift of W. R. Mayberry, Quillen-Dishner College of Medicine, East Tennessee State University, Johnson City, Tenn.

A flow diagram of the method for assay of hydroxy fatty acids of the LPS from sediments is given in Fig. 1.

Lipid extraction. Sediments up to 50 g wet weight sieved through a 500-μm screen in the field or 50 mg of lyophilized cultures were placed in a 250-ml separatory funnel and extracted with the modified one-phase chloroform-methanol Bligh and Dyer (1) extraction (32). The sediments were suspended in sufficient 50 mM phosphate buffer (pH 7.4) so that the total aqueous content of buffer plus pore water was less than 30 ml, and 75 ml of anhydrous methanol and 37.5 ml of chloroform were added. The mixture was shaken and allowed to stand for at least 2 h. After 2 h, an additional 37.5 ml each of chloroform and water were added, the mixture was shaken, and the phases were allowed to separate for at least 24 h at room temperature. The chloroform phase was drawn off through a fluted Whatman 2V filter paper and analyzed for lipids (32). After removal of the chloroform phase, the residue was transferred quantitatively to a 250-ml round-bottomed flask by using a portion of the aqueous phase. The residue was dried in vacuo on a rotary evaporator and suspended in 30 ml of 1 N HCl. After refluxing at 100°C for 5 h and cooling, the supernatant was transferred to a 125-ml separatory funnel with washes of 30, 10, and 10 ml of chloroform. The two phases were allowed to separate, and the chloroform was collected into a round-bottomed flask. The solvent was removed in vacuo and the lipid transferred to a screw-capped tube with three 2-ml portions of chloroform. The solvent was removed in a stream of nitrogen at 40°C. The samples were esterified by adding 1 ml of methanol-chloroform-concentrated HCl, 10:1:1 (vol/vol), and heating at 100°C for 1 h. After addition of 1 ml of chloroform and 1 ml of water with thorough mixing, the chloroform layer was recovered, evaporated in a stream of nitrogen, and spotted on a thin-layer chromatography (TLC) plate (Whatman K6 silica gel, 250 μm thick). After ascending chromatography in a solvent of hexane-diethyl ether, 1:1 (vol/vol) (16), the hydroxy fatty acids band (R_f, 0.4) was recovered in a Pasteur pipette with suction, and the esters were eluted from the silica gel with chloroform-methanol 1:1 (vol/vol), into screw-capped glass test tubes. The band containing hydroxy fatty acid esters was detected by using standards spotted on the outer edges of the plates as described previously (2). At this point, 413 pmol of the internal standard, hexadecanol dissolved in chloroform, were added, and all solvents were removed in a stream of nitrogen.

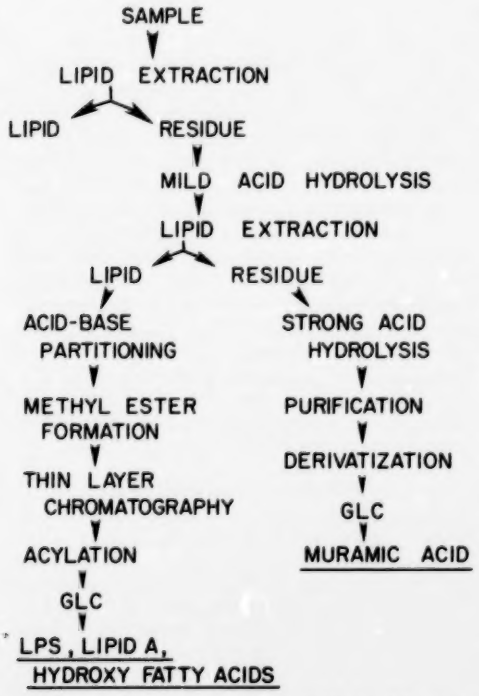

FIG. 1. Flow diagram of the hydroxy fatty acid analysis from lipid A of gram-negative bacteria.

Phenol-water extraction. The phenol-water extraction (PW) described by Westphal et al. (28) as modified by Saddler and Wardlaw (23) was utilized. Lyophilized *E. coli* (0.15 mg dry weight) were suspended in 100 ml of water at 68°C, mixed with an equal volume of 90% (wt/vol) phenol-water, and shaken for 15 min, cooled in an ice bath, centrifuged at 3,000 × *g* for 10 min, and the aqueous layer recovered. The phenol layer was reextracted with water twice. The aqueous layers were collected, dialyzed against running water, centrifuged at 105,000 × *g* for 1 h, and the pellet lyophilized. The extract was hydrolyzed in HCl and the fatty acids were purified by TLC and analyzed as described above.

Trichloroacetic acid extraction. Samples of lyophilized *E. coli* were suspended in 1.0 N trichloroacetic acid in glass centrifuge tubes and extracted as described (23). The LPS was recovered, lyophilized, hydrolyzed, and extracted with chloroform. The fatty acids were recovered, and purified by TLC as described above.

Acylations. The fatty acid methyl esters were dissolved in 0.5 ml of benzene containing 0.1 ml of heptafluorobutyric anhydride and 50 mM trimethylamine and heated for 15 min at 60°C. After cooling, 1 ml of benzene and 1 ml of water were added, and the solution was mixed on a Vortex mixer for 1 min. After

addition of 1 ml of aqueous ammonia (1% [vol/vol]), the mixing was continued for 5 min. The suspension was centrifuged, the benzene layer recovered, and the sides of the tube and surface of the water were washed three times with benzene without mixing. The benzene washes were collected, the benzene removed in a stream of nitrogen, and the acylated esters dissolved in hexane for GLC.

Hydrogenations. Fatty acid methyl esters were dissolved in hexane and hydrogenated for 1 h at 25°C at 1 atm (100 kPa) of H_2 with a catalyst of platinum(IV) oxide (Aldrich). When samples were hydrogenated, an internal standard of 80 nmol/ml of nonadecanoic methyl ester was added. The olefin esters were completely hydrogenated by this process (31).

GLC. GLC was performed on a Varian 3700 instrument with either flame ionization detectors or a ^{63}Ni electron capture detector, a model 8000 autosampler, a CDS 111 controller, and a Hewlett-Packard 3502 programmable laboratory data system. The chromatograph contained a 50-m glass capillary column (0.24 mm inside diameter) coated with the polar cyanoalkyl phenyl polysiloxane Silar 10C (Applied Science Laboratories, College Station, Pa.); it operated with splitless injection with a 0.5-min venting time, after a 2-μl injection of hexane at 42°C followed by a linear increase of 1°C/min to 190°C. The helium carrier gas flow rate was 1 ml/min, with ultrapure nitrogen at 30 ml/min as the makeup gas. The injector temperature was 220°C and the detector 250°C.

Silane derivative formation. For gas chromatography-mass spectrometry (GC-MS), the fatty acid methyl esters were trimethylsilylated by adding 0.5 ml of freshly prepared pyridine–*N,O*-bis-(trimethylsilyl)trifluoroacetamide–hexamethyldisilazane–trimethylchlorosilane (TMSi), 0.2:1:2:1 (vol/vol) (prepared by adding each reagent in the order given with mixing), heating for 15 min at 50°C, removing the solvents in a stream of nitrogen, and dissolving in hexane for GC-MS analysis.

MS. The Hewlett-Packard 5995A GC-MS was operated by using the chromatographic parameters described above after autotuning with decafluorotriphenylphosphine at an ionization potential of 70 meV. The scan speed was 690 amu/s over the range 40 to 400 amu with a 0.5-s delay. The electron multiplier potential was 1,400 V, and the recovery system was operated in the peakfinder mode.

Fatty acid designation. Fatty acids are designated as the number of carbon atoms:the number of double bonds (e.g., 14:0, 16:0), with prefixes a and i for anteisobranching and isobranching. The prefix OH indicates a hydroxyl group at the position indicated from the carboxyl end.

RESULTS

Efficiency of extraction. The recovery of 3-OH 14:0 from the lipid extracted residue of *E. coli* was examined by three extraction methods. Recoveries for each method were (μmol/g of dry weight ± standard deviation): phenol-water, 1.7 ± 0.60; trichloroacetic acid, 3.14 ± 0.27; lipid extraction after acid hydrolysis, 14.4 ± 2.0. The classical methods of LPS extraction, phenol-water or trichloroacetic acid, yielded fourfold less 3-OH 14:0 than did the proposed method involving lipid extraction followed by mild acid hydrolysis of the lipid-extracted residue. In these experiments, the phenol-water and the trichloroacetic acid extractions yielded 98 and 245% of the values reported for *E. coli*-mud mixtures by Saddler and Wardlaw (23). Of the 3-OH 14:0 from the whole bacteria, 100 ± 2.2% (n = 5) was found in the lipid-extracted residue.

Recovery of hydroxy fatty acids in the extraction procedure. The recovery of 0.42 μmol of 3-OH 14:0 and 12-OH 18:0 added to acid washed sand and extracted and purified by TLC was 95 ± 12% and 100 ± 9%, respectively. The hydrolysis of this mixture in 1 N HCl for 2 h at room temperature resulted in recoveries of 98 ± 3% of the 3-OH 14:0 and 95 ± 6% of the 12-OH 18:0. Heating the hydrolysis mixture at 100°C for 5 h did not change the recovery. The addition of lyophilized *E. coli* to sediments resulted in quantitative recovery (99 ± 2%) of the 3-OH 14:0 added to the sediments. The sediments contained about 2 nmol of 3-OH 14:0/g of dry weight (Table 1).

Sensitivity. When the heptafluorobutyric anhydride derivative of the hydroxy fatty acids was analyzed with splitless injection on glass capillary columns with the flame ionization detector, the sensitivity limit (measured in integrator counts 2.5 times the background) for 3-OH 14:0 was 4.1×10^{-12} mol. By using electron capture detection, the sensitivity was 2.26×10^{-13} mol of 3-OH 14:0.

Hydrolysis. Release of the fatty acids covalently bonded to the LPS in the lipid-extracted residue was routinely done in acid since both ester and amide-linked fatty acids might be present. Strong acid (6 N HCl) treatment resulted in poor recoveries of authentic 3-OH fatty acids so 1 N HCl was used. The acid catalyzed formation of esters with nonhydroxy fatty acids in the lipid phase reported by Wilkinson (34) did not occur with 1 N HCl and the recovery of added authentic 12-OH 18:0 and 3-OH 14:0 was quantitative.

Chromatographic analysis. The hydroxy fatty acids recovered after mild acid hydrolysis of the lipid-extracted residue of estuarine sediment are illustrated in Fig. 2. The internal standard of 413 pmol of hexadecanol was added just before derivatization with heptafluorobutyric anhydride. When hydrogenation of the methyl esters was done, an additional internal standard of 19:0 methyl ester was added. The elution times of known standards are indicated on the ordinate.

Identification of the hydroxy fatty acids. The presence of a carboxyl group in the lipids was verified by demonstrating preferential partitioning into aqueous base from petroleum ether and back into petroleum ether after acidification of

TABLE 1. Hydroxy fatty acids extracted after mild acid hydrolysis of the lipid-extracted residue of microorganisms and sediments

Component	Amount of fatty acids[a]					
	Organism (μmol/g of dry weight)				Sediment (nmol/g of dry weight)	
	E. coli	*P. fluorescens*	*P. atlantica*	*P. maltophilia*	Estuarine	Ground water
2-OH 12:0	ND[b]	1.08 (0.1)	ND	ND	ND	ND
3-OH 12:0	ND	1.23 (0.3)	0.34 (0.01)	0.70 (0.20)	0.24 (0.08)	ND
3-OH 13:0	ND	ND	ND	1.08 (0.2)	ND	ND
2-OH 14:1	ND	ND	ND	0.15 (0.04)	ND	ND
3-OH 14:0	15.5 (1.0)	0.13 (0.01)	4.28 (0.1)	0.2 (0.02)	2.26 (0.35)	0.34 (0.38)
2-OH 15:1	0.33 (0.09)	0.11 (0.01)	ND	0.12 (0.02)	0.82 (0.03)	ND
3-OH 16:0	ND	ND	ND	ND	0.66 (0.1)	0.07 (0.07)
A	0.09 (0.01)	0.07 (0.02)	ND	0.05 (0.02)	0.50 (0.12)	ND
3-OH 18:0	ND	ND	ND	ND	0.52 (0.27)	ND
B	0.03 (0.02)	0.05 (0.03)	ND	0.02 (0.01)	ND	ND
12-OH 18:0	0.15 (0.08)	ND	ND	0.02 (0.01)	0.24 (0.08)	0.004 (0.005)
Phospholipid	47.4 (5.0)	84.4 (5.3)	48.3 (6.0)	60.4 (8.9)	28 (9.0)	0.98 (0.018)

[a] Values in parentheses are standard deviations; $n = 4$.
[b] ND, Not detectable (<0.15 nmol/g of dry weight).

the aqueous phase. This procedure was used in the purification of samples from the environment. There was quantitative recovery of 3-OH 14:0 in this procedure.

Although the heptafluorobutyric anhydride derivatives gave greater sensitivity and were much less damaging to the polar stationary phases of the capillary columns, the TMSi ether derivatives gave a more definitive fragmentation pattern with GC-MS. All of the hydroxy fatty acids show very little M^+ but a prominent M-15 (loss of CH_3 from TMSi) with base fragments at *m/e* 73 $[Si^+(CH_3)_3]$ or 75 $[SiO^+H(CH_3)_2]$ and prominent *m/e* 89 $[SiO^+(CH_3)_3]$ (7).

The TMSi ethers of the 2-OH esters show the definitive M-59 $[RCH{=}O^+Si(CH_3)_3]$ (7) as well as major fragments at *m/e* 103 $[CH_2{=}O^+Si(CH_3)_3]$ and *m/e* 129 $[CH_2{=}CHCHO^+Si(CH_3)_3]$.

The TMSi ethers of the 3-OH fatty acid esters show the definitive fragment at *m/e* 175 $[CH_3CO_2CH_2CH{=}O^+Si(CH_3)_2]$ plus *m/e* 133 (rearrangement with loss of C_2H_2O from 175), *m/e* 159 $[CH_3CO_2CH{=}CHO^+Si(CH_3)_2]$, and *m/e* 131 (loss of CO from *m/e* 159) (7).

The ω-OH fatty acids show intense peaks at M-15, M-47 $(CH_3)_2SiO^+RCHCH{=}C{=}O$, and M-31 $(CH_3)_3SiO(CH_2)_nC^+{=}O$. None were detected in these samples.

The TMSi ethers of hydroxy fatty acid methyl esters with the OH group near the middle of the chain show prominent *m/e* 175 plus $(CH_2)_{n-1}$ where *n* is the number of carbons between the carboxyl and hydroxyl carbons.

Anteisobranched fatty acids often show M-57 > M-43 $[M\text{-}(C_4H_9) > M\text{-}(C_3H_7)]$. This is reversed in isobranched esters. However, the position of the branching is more consistently determined by the chromatographic elution volume. For each homologue, the elution order is isobranched, anteisobranched, and normal on the polar columns utilized in this study.

Two components present in significant amounts could not be identified. Component A eluted between 16:0 and 18:0 and component B eluted between the 2-OH 18:0 and 3-OH 18:0.

Unsaturation was demonstrated by fragmentography and the shift on catalytic hydrogenation. The positions of unsaturation were not determined.

The criteria for identification are listed in Table 2.

Distribution of bound hydroxy fatty acids. The data of Table 1 show the distribution of the bound hydroxy fatty acids and lists the extractable phospholipid content of each system. The pseudomonads examined appear to be distinctly different from the *E. coli*, which is in agreement with previous studies (21, 28). The sediments from both the surface and subterranean locations appear to have a bound hydroxy fatty acid composition different from any monoculture thus far examined. Monocultures of *B. subtilis* and *C. welchii* yielded no detectable (<0.01 nmol/g of dry weight) acid labile hydroxy fatty acids from the lipid extracted residue.

DISCUSSION

Specificity of the assay. The analysis of the LPS-derived hydroxy fatty acids was a convenient and sensitive method for estimating the biomass of the gram-negative bacteria that contain lipid A in environmental samples. This analysis was extremely valuable in the analysis of the sparse microbiota of ground water sediments. Since there is a relatively rapid turnover

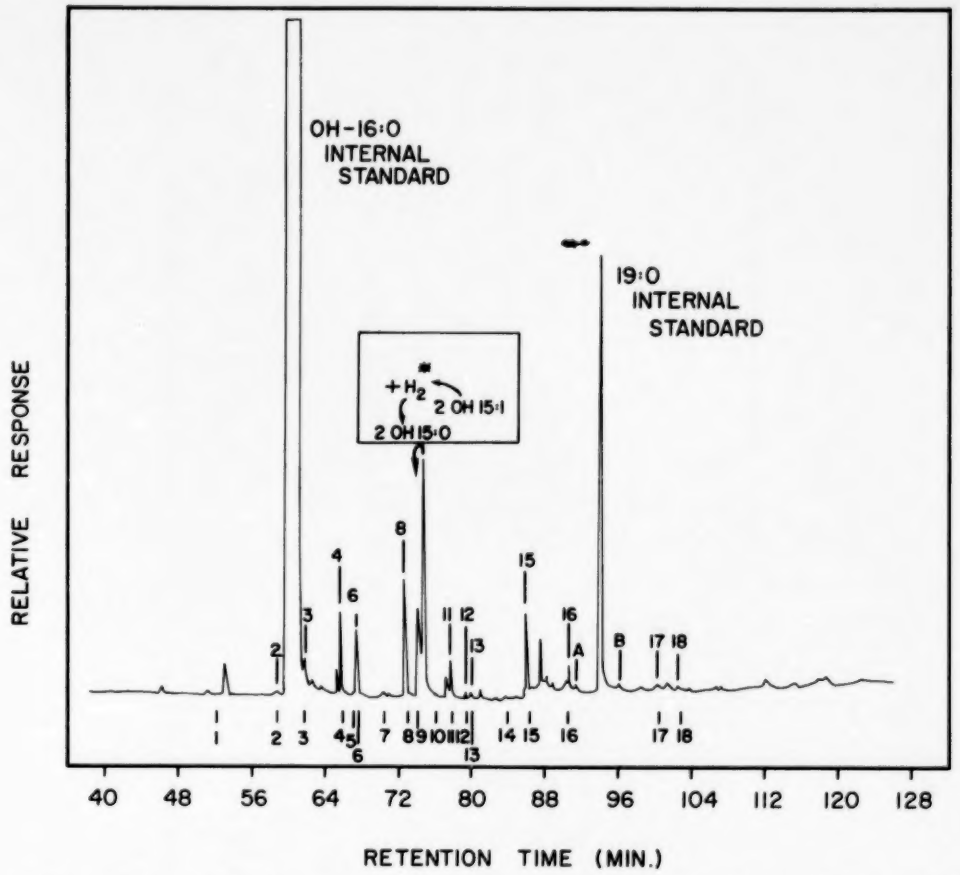

FIG. 2. Capillary gas-liquid chromatogram of the heptafluorobutyrate esters of a pooled sample of the hydroxy fatty acid methyl esters rendered extractable after mild acid hydrolysis of the lipid-extracted residue of the microbiota of estuarine sediments. The positions of authentic hydroxy fatty acids are indicated below the abscissa. Elution volumes of authentic standards are indicated as 1, 2-OH 12:0; 2, 3-OH 12:0; 3, 3-OH 14:0; 4, 2-OH 13:0; 5, 3-OH 14:1; 6, 3-OH i 14:0; 7, 3-OH a 14:0; 8, 3-OH 14:0; 9, 2-OH 15:0; 10, 3-OH i 15:0; 11, 3-OH a 15:0; 12, 3-OH 15:0; 13, 2-OH 16:0; 14, 3-OH i 16:0; 15, 3-OH 16:0; 16, 3-OH i 17:0; 17, 3-OH 18:0; 18, 12-OH 18:0. Internal standards of hexadecanol (OH-16:00) and nonadecanoic methyl ester (19:0) are indicated. The shift in position of the 2-OH 15:1 after catalytic hydrogenation in hexane is indicated by the arrow and the H_2*.

of LPS in nature (23), the measurement is probably indicative of only the viable gram-negative population.

With few exceptions (35), all bacterial LPS contains lipid A, but there is wide variation in the detailed composition among different organisms (21, 35). The fatty acid composition in the lipid A can reflect the composition of the growth medium in some organisms (35). The fatty acids of the LPS represent the major component of the lipid A (23). However, the lipid A hydroxy fatty acids appear to be the least variable of the LPS components with changes in growth conditions (23).

Ketodeoxyoctonate is unique to LPS and is widely distributed among gram-negative organisms. However, the amount of ketodeoxyoctonate in various LPS preparations can be quite variable and dependent upon growth conditions (8). The assay of ketodeoxyoctonate by the thiobarbituric acid colorimetric test is subject to interference by materials in the LPS as well as other cellular components (23).

Although the *Limulus* amebocyte lysate coagulation assay has been utilized widely in water column samples (24) and can be extremely sensitive (13), it presents some problems. There is a variability in results between operators with the

TABLE 2. Identification of the hydroxy fatty acids extracted after mild acid hydrolysis of the lipid-extracted residue of microorganisms and sediments

Component	*m/e* (relative abundance)					MS fragments [*m/e* (relative intensity)] of TMSi methyl esters
	M-15	M-31	M-43	M-47	M-59	
2-OH 12:0[a]	287 (9.6)	—[b]	259 (5)	—	243 (87)	73 (100), 75 (36), 89 (48), 103 (32), 129 (12), 159 (5)
3-OH 12:0[a]	287 (60)	271 (2)	—	255 (3)	243 (12)	73 (100), 75 (62), 89 (86), 103 (15), 129 (9), 131 (27), 133 (32), 159 (35), 175 (75)
3-OH 13:0[a]	301 (48)	285 (2)	273 (0.2)	269 (2)	257 (1)	73 (100), 75 (88), 89 (83), 103 (23), 129 (7), 131 (9), 133 (31), 159 (34), 175 (70)
2-OH 14:1[a]	313 (73)	—	—	—	—	73 (55), 75 (100), 89 (27), 103 (47), 129 (6), 131 (9), 159 (9), 175 (13)
3-OH 14:0[a]	315 (61)	313 (2)	—	297 (3)	285 (1)	73 (91), 75 (64), 89 (87), 103 (6), 129 (8), 131 (3), 133 (38), 159 (44), 175 (100)
2-OH 15:1[a]	327 (5)	311 (1)	—	—	—	75 (30), 87 (66), 89 (3), 103 (3), 129 (10), 131 (3), 133 (3), 143 (19), 157 (4), 171, (5), 175 (1)
3-OH 16:0[a]	343 (24)	327 (3)	—	311 (3)	—	73 (35), 75 (33), 89 (26), 103 (7), 129 (13), 131 (24), 133 (20), 159 (23), 175 (36)
A	—	—	—	—	—	73 (15), 75 (39), 87 (64), 97 (26), 112 (15), 129 (13), 143 (15), 156 (10), 162 (8), 199 (10), 213 (10), 298 (10), 407 (11)
B	—	—	—	—	—	75 (6), 87 (29), 129 (5), 131 (55), 133 (4), 150 (20), 159 (1), 166 (4), 175 (1), 180 (6), 222 (6), 264 (10), 296 (2)
12-OH 18:0[a]	—	355 (1)	—	311 (1)	—	73 (24), 75 (81), 89 (2), 103 (17), 129 (8), 131 (22), 133 (4), 159 (5), 175 (2)

[a] Indicates cochromatography with an authentic standard.
[b] —, No fragment.

same samples (27), a lack of specificity to some bacterial products (7, 33), and since a change in turbidity is the measure of activity, the microbes attached to sediments must be quantitatively recovered from the sediments for assay. Free endotoxin, released by cells without lysis, can complicate the *Limulus* amebocyte lysate assay when used to diagnose clinically important infections (22). This should not be a problem with the hydroxy fatty acid assay proposed here, since the free LPS in nature has a rapid turnover and only bound LPS is measured (21). In addition, the *Limulus* amebocyte lysate assay does not provide data on the composition of the LPS hydroxy fatty acids.

In the present method, it is possible that low-molecular-weight fragments of LPS can be extracted with the preliminary lipid extraction. This is most likely a problem in the extraction of wet samples (W. R. Mayberry, personal communication). Losses of LPS in the preliminary lipid extraction would yield an underestimation of the gram-negative bacterial biomass. However, our method produced yields of bound hydroxy fatty acids that were 4 to 10 times larger than other extraction methods.

Hydroxy fatty acids are widely distributed in nature (3, 6, 12, 26). In sediments, the bound, acid-labile hydroxy fatty acids form the largest pools (5) and their occurrence correlates with environments enriched in bacteria (4). Plants contain an incredible assortment of lipids that could complicate this assay. Extractable lipids with 2-hydroxy fatty acids of chain lengths between 12 and 26 carbon atoms, 3-hydroxy fatty acids of 16, 18 and 20 carbon atoms, ω-hydroxy fatty acids (both saturated and unsaturated) of 12 to 30 carbon atoms, midchain hydroxy fatty acids in which HO—$C(CH_2)_yCOOH$ structures have y values from 5 to 8, diols, and polyols as well as mixes of hydroxy-oxy or hydroxy-epoxy fatty acids have been reported (12, 14, 15). In plants these fatty acids are minor components of the seed oils or fragments of the cutin or suberin hydrolyses and are most often extractable with the lipids. Nonextractable hydroxy fatty acids typical of those from plants have not complicated the assay of marine and estuarine sediments.

Community structure. The composition of the hydroxy fatty acids can be utilized to give additional insight into the community structure as in Table 1. From the LPS hydroxy fatty acid analysis, the gram-negative organisms in the estuarine and groundwater aquifer sediments indicate a complex mixture of a wide variety of organisms. Since this method utilizes purification and assay by GLC, it should be possible to utilize enrichment of ^{13}C with analysis by GC-MS to follow rates of synthesis and turnover in the specific components of the community.

Muramic acid can be determined by the application of an additional acid hydrolysis of the residue extracted for the LPS-lipid A hydroxy fatty acids (see Fig. 1) (Findlay et al., in press). This can provide another estimate of the community structure. The estuarine and groundwater aquifer sediments used for the data of Table 1 contained 860 ± 60 and 2.1 ± 1.2 nmol of muramic acid/g of dry weight, respectively. The ratios of muramic acid to the 3-OH 14:0 from the lipid A of the estuarine and aquifer sediments were 380 and 14, respectively, indicating the groundwater aquifer contained a larger proportion of procaryotes with the 3-OH 14:0 type of LPS than the estuarine sediments.

ACKNOWLEDGMENTS

We thank W. A. Corpe and T. G. Tornabene for cultures of *Pseudomonas* and W. R. Mayberry for a collection of authentic hydroxy fatty acid standards.

This work was supported by contract N00014-75-C-0201 from the Department of the Navy, Office of Naval Research, Ocean Science and Technology Detachment; contract 04-7-158-4406 from the National Oceanic and Atmospheric Administration, Office of Sea Grant, Department of Commerce; contract 80-7321-02 from the U.S. Environmental Protection Agency, administered by the Gulf Breeze Environmental Research Laboratory, Gulf Breeze, Fla., grant NAG2-149 from the Advanced Life Support Office, National Aeronautics and Space Administration, and grants OCE 80-19757 and DEB 78-18401 from the Biological Oceanography Program of the National Science Foundation.

LITERATURE CITED

1. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of lipid extraction and purification. Can. J. Biochem. Physiol. **35**:911–917.
2. **Bobbie, R. J., and D. C. White.** 1980. Characterization of benthic microbial community structure by high-resolution gas chromatography of fatty acid methyl esters. Appl. Environ. Microbiol. **39**:1212–1222.
3. **Boon, J. J., F. de Lange, P. J. W. Schuyl, J. W. de Leeuw, and P. A. Schenck.** 1977. Organic geochemistry of Walvis Bay diatomaceous ooze. II. Occurrence and significance of the hydroxy fatty acids, p. 255–273. *In* R. Campos and J. Goni (ed.) Advances in organic geochemistry 1975. Empresa Nacional de Adaro de Investigaciones Mineras, S. A. Madrid.
4. **Brooks, P. W., G. Eglinton, S. J. Gaskell, D. J. McHugh, J. R. Maxwell, and R. P. Philip.** 1976. Lipids of recent sediments, part I. Straight chain hydrocarbons and carboxylic acids of some temperate lacustine and subtropical lagoonal tidal flat sediments. Chem. Geol. **18**:21–38.
5. **Cranwell, P. A.** 1981. The stereochemistry of 2- and 3-hydroxy fatty acids in a recent lacustine sediment. Geochim. Cosmochim. Acta **45**:547–552.
6. **Downing, D. T.** 1961. Naturally occurring aliphatic hydroxyacids. Rev. Pure Appl. Chem. **11**:196–211.
7. **Eglinton, G., D. H. Hunneman, and A. McCormick.** 1968. Gas chromatographic-mass spectrometric studies of long chain hydroxy acids III. The mass spectra of the methyl esters, trimethylsilylethers of aliphatic hydroxy acids. A facile method of double bond location. Org. Mass Spec. **1**:593–611.
8. **Ellwood, D. C.** 1970. The distribution of 2 keto-3-deoxyoctonoic acid in bacterial walls. J. Gen. Microbiol. **60**:373–380.
9. **Elvin, R. J., and S. M. Wolff.** 1973. Non-specificity of the *Limulus* amebocyte lysate test: positive reactions with polynucleotides and proteins. J. Infect. Dis. **128**:349–352.
10. **Fazio, S. D., W. R. Mayberry, and D. C. White.** 1979.

Muramic acid assay in sediments. Appl. Environ. Microbiol. **38**:349–350.

11. **Fazio, S. D., D. J. Uhlinger, J. H. Parker, and D. C. White.** 1982. Estimations of uronic acids as quantitative measures of extracellular and cell wall polysaccharide polymers from environmental samples. Appl. Environ. Microbiol. **43**:1151–1159.
12. **Harwood, J. L.** 1980. Plant acyl lipids: structure, distribution, and analysis, p. 2–56. *In* P. K. Stumpf and E. E. Conn (ed.), Biochemistry of plants: a comprehensive treatise, vol. 4. Academic Press Inc., New York.
13. **Jorgeusen, J. H., J. C. Lee, and H. R. Pahren.** 1976. Rapid detection of bacterial endotoxins in drinking water and renovated wastewater. Appl. Environ. Microbiol. **32**:347–351.
14. **Kolattukudy, P. E.** 1980. Cutin, suberin, and waxes, p. 571–645. *In* P. K. Stumpf and E. E. Conn (ed.), The biochemistry of plants: a comprehensive treatise, vol. 4. Academic Press Inc., New York.
15. **Kolattukudy, P. E.** 1981. Structure, biosynthesis and biodegradation of cutin and suberin. Annu. Rev. Plant Physiol. **32**:539–567.
16. **Mayberry, W. R.** 1980. Hydroxy fatty acids in *Bacteroides* species: D-(−)-3-hydroxy-15-methylhexadecanoate and its homologs. J. Bacteriol. **143**:582–587.
17. **Mayberry, W. R., D. W. Lambe, Jr., and K. P. Ferguson.** 1982. Identification of *Bacteroides* species by cellular fatty acid profiles. Int. J. Syst. Bacteriol. **32**:21–37.
18. **Moriarty, D. J. W.** 1977. Improved method using muramic acid to estimate biomass of bacteria in sediments. Oecologia **26**:317–323.
19. **Moriarty, D. J. W.** 1980. Problems in the measurement of bacterial biomass in sandy sediments, p. 131–139. *In* P. A. Trudinger, M. R. Walter, and B. J. Ralph (ed.), Biogeochemistry of ancient and modern environments. Australian Academy of Science, Canberra.
20. **Moriarty, D. J. W., and A. C. Hayward.** 1982. Ultrastructure of bacteria and the proportions of gram negative bacteria in marine sediments. Microb. Ecol. **8**:1–12.
21. **Reitschel, E. T., C. Galanos, and O. Luderitz.** 1975. Structure, endotoxicity, and immunogenicity of the lipid A component of bacterial lipopolysaccharides, p. 307–314. *In* D. Schlessinger (ed.), Microbiology—1975. American Society for Microbiology, Washington, D.C.
22. **Russell, R. R. B.** 1976. Free endotoxin—a review. Microbios Lett. **2**:125–135.
23. **Saddler, J. N., and A. C. Wardlaw.** 1980. Extraction, distribution, and biodegradation of bacterial lipopolysaccharides in estuarine sediments. Antonie van Leeuwenhoek J. Microbiol. Serol. **46**:27–39.
24. **Sullivan, J. D., Jr., and S. W. Watson.** 1974. Factors affecting the sensitivity of *Limulus* lysate. Appl. Environ. Microbiol. **28**:1023–1026.
25. **Tornabene, T. G., and S. L. Peterson.** 1978. *Pseudomonas maltophilia* identification of the hydrocarbons, glycerides and glycoproteins of cellular lipids. Can. J. Microbiol. **24**:525–532.
26. **Volkman, J. K., R. B. Johns, F. T. Gillan, and G. J. Perry.** 1980. Microbial lipids of an intertidal sediment I. Fatty acids and hydrocarbons. Geochim. Cosmochim. Acta **44**:1133–1143.
27. **Watson, S. W., T. J. Novitsky, H. L. Quinby, and F. W. Valois.** 1977. Determination of bacterial number and biomass in the marine environment. Appl. Environ. Microbiol. **33**:940–946.
28. **Westphal, O., O. Luderitz, and F. Bister.** 1952. Uber bakterielle Reizstoffe I mit Reindarstellung eines Polysaccharid-Pyrogens aus *Bacterium coli*. Z. Naturforsch. Teil B **7b**:537–548.
29. **White, D. C., R. J. Bobbie, J. S. Herron, J. D. King, and S. J. Morrison.** 1979. Biochemical measurements of microbial mass and activity from environmental samples, p. 69–81. *In* J. W. Costerton and R. R. Colwell (ed.), Native aquatic bacteria: enumeration, activity and ecology. ASTM STP 695, American Society for Testing and Materials, Philadelphia.
30. **White, D. C., R. J. Bobbie, J. S. Nickels, S. D. Fazio, and W. M. Davis.** 1980. Nonselective biochemical methods for the determination of fungal mass and community structure in estuarine detrital microbiota. Bot. Mar. **23**:239–250.
31. **White, D. C., and R. H. Cox.** 1967. Identification and localization of the fatty acids in *Haemophilus parainfluenzae*. J. Bacteriol. **93**:1079–1088.
32. **White, D. C., W. M. Davis, J. S. Nickels, J. D. King, and R. J. Bobbie.** 1979. Determination of the sedimentary microbial biomass by extractable lipid phosphate. Oecologia **40**:51–62.
33. **Wildfeuer, A., B. Heymer, K. H. Schleifer, and O. Haferkamp.** 1974. Investigations on the specificity of the Limulus test for detection of endotoxin. Appl. Environ. Microbiol. **28**:867–871.
34. **Wilkinson, S. G.** 1974. Artifacts produced by acidic hydrolysis of lipids containing 3-hydroxy acids. J. Lipid Res. **15**:181–182.
35. **Wilkinson, S. G.** 1977. Composition and structure of bacterial lipopolysaccharides, p. 99–175. *In* I. W. Sutherland (ed.), Surface carbohydrates of the prokaryotic cell. Academic Press Inc., New York.